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Genetic diversity and relationship among faba bean (*Vicia faba* L.) germplasm entries as revealed by TRAP markers

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Abstract

Target region amplification polymorphism markers were used to assess the genetic diversity and relationship among 151 worldwide collected faba bean (*Vicia faba* L.) entries (137 accessions maintained at the USDA–ARS, Pullman, WA, 2 commercial varieties and 12 elite cultivars and advanced breeding lines obtained from Link of Georg-August University, Germany). Twelve primer combinations (six sets of polymerase chain reaction) amplified a total of 221 markers, of which 122 (55.2%) were polymorphic and could discriminate all the 151 entries. A high level of polymorphism was revealed among the accessions with an estimated average pairwise similarity of 63.2%, ranging from 36.9 to 90.2%. Cluster analysis divided the 151 accessions into five major groups with 2–101 entries each and revealed a substantial association between the molecular diversity and the geographic origin. All 101 accessions in Group V are originated from China and 13 of the 15 accessions in Group II were from Afghanistan. Thirty-two individual plants were sampled from two entries to assess the intra-accession variation. It was found that the advanced inbred line (Hiverna/5-EP1) had very little variation (5.0%), while the original collection (PI 577746) possessed a very high amount of variation (47.1%). This is consistent with the previous reports that faba bean landraces have a high level of outcrossing in production fields and thus contain larger amount variation within each landrace. One implication of this observation for germplasm management is that a relatively larger population is needed in regeneration to mitigate the possible loss of genetic variation due to genetic drift.

Keywords: germplasm management; target region amplification polymorphism (TRAP)

Introduction

Faba bean (*Vicia faba* L.), also referred to as broad bean, field bean, tick bean, windsor bean and horse bean, is one of the oldest crops grown by humans and is a valuable protein-rich food and animal feed (Link *et al*., 1995; Duc, 1997; Zong *et al*., 2009). Large-seeded faba bean grain is usually used as food, while medium-sized grain is used as food and feed, with small-sized grain mainly used as feed (Redden *et al*., 2007). In production, faba bean can be grown in rotation with cereal crops for improving soil physical condition, breaking disease cycles and controlling weeds (Duc, 1997; Murray *et al*., 1998). China is the leading faba bean producer with 43% of the world's faba bean crop, followed by Ethiopia, Egypt, France and Australia (FAO, 2006). However, in spite of its great potential for being an important protein source in many countries, its area of cultivation has been

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decreasing over the years (Torres *et al.,* 1993). This acreage reduction is mainly attributed to the unstable yielding ability of faba bean.

Knowledge of the genetic diversity and the relationships among conserved germplasm collections of a crop is essential and of critical importance in establishing, managing and ensuring a long-term success of crop improvement programs (Gwak, 2008; Ma *et al.*, 2009). In faba bean, genetic diversity among collected germplasm accessions has been delineated by various kinds of genetic marker systems, including isozyme (Käser and Steiner, 1983; Mancini *et al.*, 1989), random amplified polymorphic DNA (RAPD) (Torres *et al.,* 1993; Link *et al.*, 1995), restriction fragment length polymorphism (Torres *et al.,* 1993), inter-simple sequence repeat (ISSR; Terzopoulos and Bebeli, 2008) and amplified fragment length polymorphism (AFLP; Zeid *et al.,* 2003; Zong *et al.*, 2009). These approaches have been instrumental in explaining the genetic diversity and relationships among accessions in faba bean *ex situ* germplasm collections. Zeid *et al.* (2003) reported the genetic diversity of 79 elite varieties of European, North African and Asian origin. Using eight AFLP primer combinations, they amplified 477 polymorphic fragments that could verify several known pedigree relationships and support available information on the history of faba bean dispersal and cultivation in the studied regions. Zeid *et al.* (2003) also found that the Asian lines were distinct as a group using both unweighted pair group method with arithmetic mean (UPGMA)-based clustering and principal coordinate analysis. A remarkable degree of intrapopulation diversity up to 0.676 was reported by Terzopoulos and Bebeli (2008) in their faba bean collection. More recently, Zong *et al.* (2009) reported the genetic diversity among Chinese and global winter faba bean germplasm assessed with AFLP. It was found that the 204 Chinese faba bean accessions were separated from the 39 accessions of various geographical origins.

Target region amplification polymorphism (TRAP) is a simple polymerase chain reaction (PCR)-based marker technique, which uses available DNA sequence information to detect the genetic variation at the DNA level (Hu and Vick, 2003). TRAP uses a fixed primer designed against the known DNA gene sequence and pairs it with arbitrary primers that target the intron or exon regions with an AT-rich or GC-rich core (Li and Quiros, 2001) to amplify the DNA fragments. Published studies applying TRAP to lettuce (Hu *et al.*, 2005), sugarcane (Alwala *et al.*, 2006), Geranium (Palumbo *et al.*, 2007), spinach (Hu *et al.,* 2007) and sunflower (Yue *et al.*, 2009) have suggested that TRAP is a useful marker system for germplasm diversity assessments. The objective of this study was to evaluate the usefulness of TRAP in revealing genetic diversity at both population and individual levels. The resulting information will be useful for improving faba bean germplasm management and promoting faba bean germplasm utilization.

Materials and methods

Plant materials

One hundred fifty-one faba bean entries were used in this study. The 137 accessions maintained at the USDA–ARS Western Regional Plant Introduction Station included 107 accessions from China, 15 from Afghanistan, 7 from Germany, 6 from Bulgaria, 4 from Nepal, 3 from France, 2 from each of the following three countries, Finland, Hungary and United Kingdom, and one from Poland. The detailed information of these 137 accessions is listed in supplementary Table S1 (available online only at http://journals.cambridge.org). The other 12 entries obtained from Professor W. Link (Georg-August University, Germany) were either cultivars or advanced breeding lines. Two vegetable-type commercial varieties were also included in this study. The names and origins of individual entry are shown in Fig. 1.

Genomic DNA extraction

Genomic DNA was extracted from the youngest leaves of 4-week-old plants grown in the field at Washington State University Whitlow farm, Pullman, WA. DNA was extracted using the DNeasy 96 Plant Kit (Qiagen, Valencia, CA, USA), employing the supplier's protocols, and quantified using a Fluoroskan Ascent FL (Thermo scientific, Rockford, IL, USA). To measure the genetic diversity among accessions, DNA was isolated from a bulk of four random plants from each entry. For assessment of intra-accession genetic variation, DNA was extracted from 16 individual plants from each of the two entries: Hiverna/5-EP1 and PI 577746.

TRAP marker generation

Four fixed primers and four arbitrary primers were used (Table 1). All the four arbitrary primers and one of the fixed primers which had been used for other fingerprinting projects (Hu *et al.*, 2005; Miklas *et al.,* 2006; Yue *et al.*, 2009) and three fixed primers with prefix 'MIR' were designed against the micro RNA sequences in *Arabidopsis thaliana* (Maher *et al.,* 2006) with the web-based PCR primer designing programme 'Primer 3',

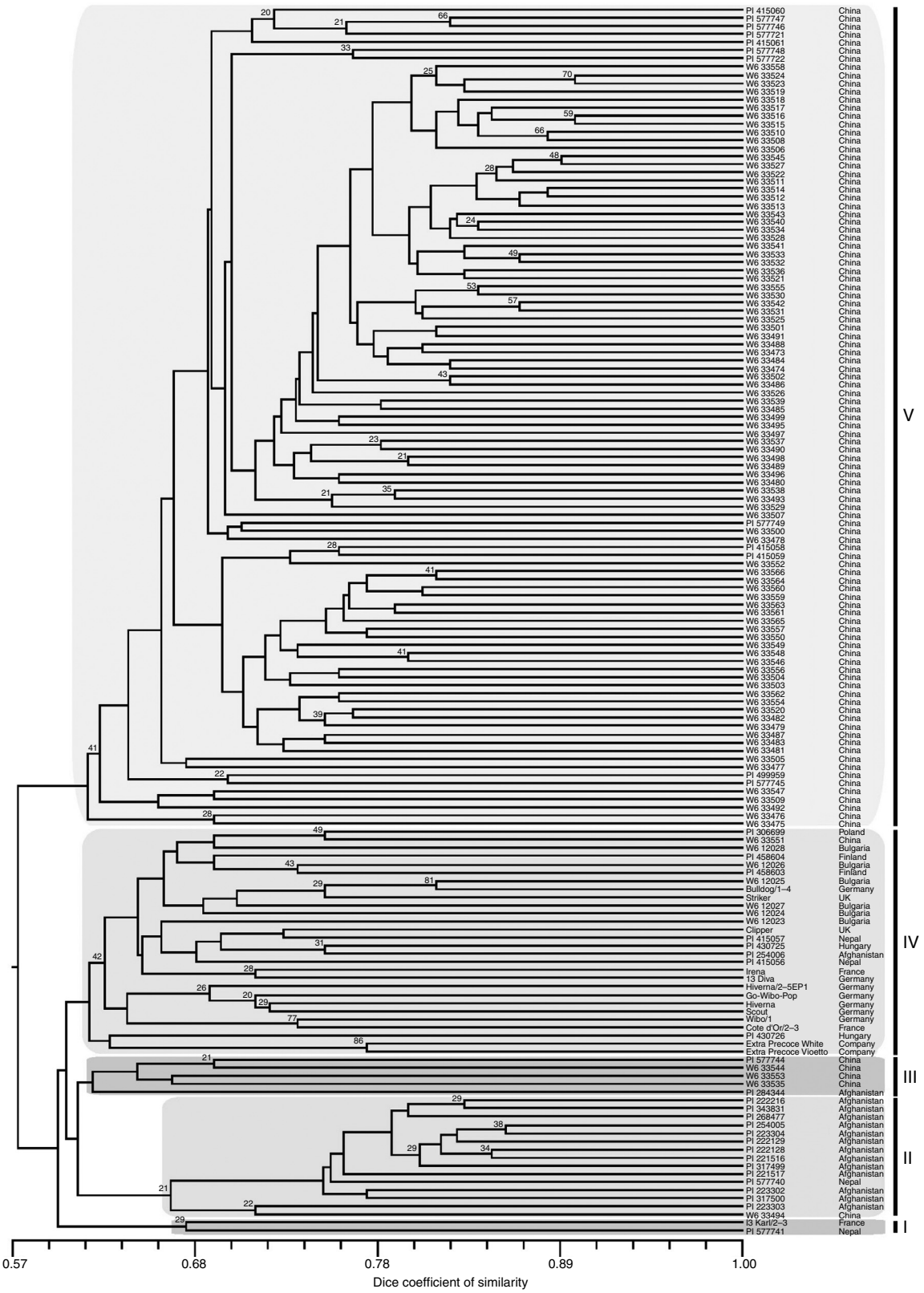

Fig. 1. A dendrogram of the 151 faba bean entries based on 122 polymorphic TRAP markers. The names of countries from which the entry was collected were denoted on the right.

Table 1. Primer names and sequences used in the current study

Primer name	Sequences (5′–3′)
Fixed primers	
B14G14B	AAT CTC AAG GAC AAA AGG
MIR 156A	GAT CTC TTT GGC CTG TC
MIR 157B	GAT CAT TGT CCA GAT TC
MIR 159A	GAT CCT TGG TTC TTT GG
Arbitrary primers	
Sa4-700	TTA CCT TGG TCA TAC AAC ATT
Sa12-700	TTC TAG GTA ATC CAA CAA CA
Ga3-800	TCA TCT CAA ACC ATC TAC AC
Ga5-800	GGA ACC AAA CAC ATG AAG A

http://frodo.wi.mit.edu/primer3/ (Rozen and Skaletsky, 2000). Six sets of PCR with 12 primer combinations were run with all the DNA samples (Table 2).

TRAP amplification was carried out using the protocol of Hu *et al.* (2007). Briefly, PCR amplification was in a total volume of 10 μl containing 1 μl of genomic DNA (10 ng/μl), 0.2 μl of the fixed primer (10 pmol/μl), 0.2 μl each of 700- and 800-IR dye-labelled arbitrary primers (1 pmol/μl), 0.8 μl of dNTP (2.5 mM), 0.3 μl of $MgCl_2$ (50 mM), 1.0 μl of 10 × PCR buffer and 0.2 μl of Taq polymerase (5 unit/μl; Bioline, Taunton, MA, USA). Conditions of the PCR amplification were as follows: 94°C (2 min), followed by 5 cycles at 94°C (45 s), 40°C (45 s), 72°C (60 s), then 35 cycles at 94°C (45 s), 50°C (45 s) and 72°C (60 s) and final extension at 72°C for 7 min. PCR was carried out in GenAmp 9700 thermal cyclers (Applied Biosystem, CA, USA).

The amplified products were separated with a 6.5% polyacrylamide sequencing gel in a Li-Cor DNA Sequencer (Li-Cor Biosciences, NE, USA) using protocols recommended by the manufacturer. Electrophoresis was conducted at 1500 V for 3 h and the images collected were manually scored.

Data analysis

Each amplified fragment was treated as a unit character and scored as a binary code 1 and 0 for presence and absence, respectively. Only the prominent bands were scored for data reliability. The data were analyzed with NTSYSpc, Numerical Taxonomy and Multivariate Analysis System version 2.11 (Exeter Software, New York, NY, USA). The Dice's coefficient (Dice, 1945) and the SIMQUAL procedure in the NTSYS pc software were used to calculate the pairwise genetic similarity ($GS = 2a/(2a + b + c)$) matrices. The GS matrices were then used to construct the dendrogram with the UPGMA algorithm (Rohlf, 2000), employing the sequential, agglomerative, hierarchical and nested clustering procedure (Sneath and Sokal, 1973). Bootstrap values were calculated with 100 replications using the software package 'WINBOOT'

Table 2. Distribution of TRAP markers amplified by different PCR and primer combinations among 151 faba bean entries

PCR	Primer combination	Total number of fragments	Polymorphic fragments	Polymorphism (%)	Shannon's information index
F01		39	19	48.7	0.148
	B14G14B + Sa12-700	24	13	54.2	0.165
	B14G14B + Ga5-800	15	6	40.0	0.122
F02		44	28	63.6	0.186
	B14G14B + Sa4-700	37	25	67.6	0.188
	B14G14B + Ga3-800	7	3	42.9	0.176
F03		44	21	47.7	0.128
	MIR159A + Sa12-700	31	16	51.6	0.144
	MIR159A + Ga5-800	13	5	38.5	0.090
F04		39	25	64.1	0.175
	MIR159A + Sa4-700	23	16	69.6	0.208
	MIR159A + Ga3-800	16	9	56.3	0.128
F05		16	6	37.5	0.104
	MIR156A + Sa12-700	9	5	55.6	0.135
	MIR156A + Ga5-800	7	1	14.3	0.064
F06		39	23	59.0	0.156
	MIR157B + Sa4-700	27	17	63.0	0.163
	MIR157B + Ga3-800	12	6	50.0	0.139
Total		221	122		
Mean					
	Per PCR	36.8	20.3	53.4	0.150
	Per primer combination	18.4	10.2	50.3	0.144

developed at International Rice Research Institute (Yap and Nelson, 1996). In addition, GenAlEx version 6.1 (Peakall and Smouse, 2006) was used to measure the Shannon's information index ($I = -1 \times (p) \times \mathrm{Ln}(p) \times q \times \mathrm{Ln}(q)$) and percentage of polymorphism.

Results

TRAP amplification profiles

All the 12 primer combinations worked successfully in the amplification of recordable fragments. The number of fragments amplified by each primer combination ranged from 7 (B14G14B + Ga3-800 and MIR156A + Ga5-800) to 37 (B14G14B + Sa4-700) and the sizes of the amplified fragments ranged from 100 to 850 base pairs (Table 2). A total of 221 amplicons were scored, and 99 fragments (44.8%) were monomorphic, whereas 122 (55.2%) fragments were polymorphic among the entries. For each primer combination, an average of 18 fragments was scored with 10 being polymorphic (Table 2). The highest level of polymorphism (69.6%) was obtained from primer combination MIR159A + Sa4-700, whereas the lowest 14.3% was obtained from primer combination MIR156A + Ga5-800. These results showed that the TRAP marker system is applicable to faba bean germplasm fingerprinting and that the efficiency of revealing polymorphism varies greatly among primer combinations.

Genetic similarity among entries

The differences among accessions at the DNA level were determined by comparing the GS coefficients for a total of 11,325 pairwise comparisons. Being estimated from the 122 informative markers, the average pairwise GS was 63.2% ranging from 36.9 to 90.2%. About 95.7% of the pairwise comparisons among faba bean entries exhibited GS greater than 50%, whereas less than 4.3% showed GS lower than 50% (Fig. 2). The top two most closely related pairs shared a GS of 90.2%, namely 'W6 33523 and W6 33524' and 'W6 33151 and W6 33516'. The least related pair, 'Herverna/5-EP1 and W6 33517', shared a GS of only 36.9%.

Genetic relationship among accessions

Based on the similarity coefficient matrix, a dendrogram was constructed using UPGMA to depict the interrelationships among the entries. As shown in Fig. 1, these 151 entries were divided into five major groups at the GS level of 0.618. This analysis revealed a substantial association between molecular diversity and geographic origin of the entries. The striking example is that all the 101 accessions in Group V were originated from China. Group II was formed by 13 entries from Afghanistan, 1 entry from China and 1 from Nepal. Group III was also composed by four Chinese accessions and an Afghanistan accession. Group I was constituted by two entries, 'I3 Karl/2-3' and 'PI577741' from France and Nepal, respectively. Group IV contained 28 accessions: 22 accessions were from European countries, 4 accessions were from Asian countries (Afghanistan, China and Nepal) and 2 were commercial vegetable-type varieties.

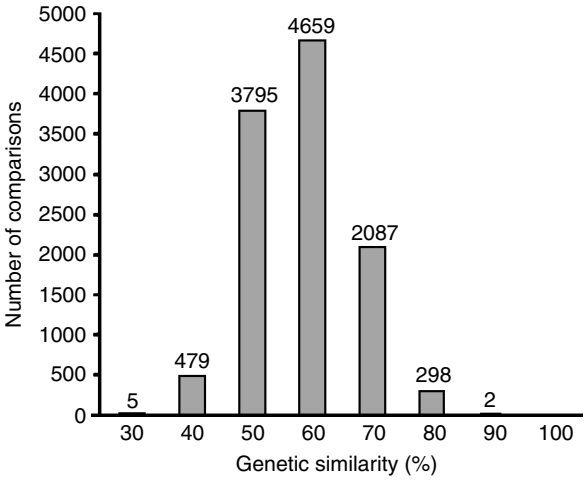

Fig. 2. A histogram depicting the distribution of 11,325 pairwise genetic similarity coefficients among 151 faba bean germplasm entries.

Intra-accession genetic diversity

Two entries ('Hirverna/5-EP1' and 'PI 577746') were used to assess intra-accession genetic variation. The same six primer combinations were run on the DNA samples prepared from 16 individual plants from each entry. The amplification profiles revealed a significant difference between these two entries. Figure 3 shows the fragments amplified by primer combination MIR159A + Sa4-700. As expected, the original collection, PI 577746, possessed a high level of genetic variation while the advanced inbred line Hirverna/5-EP1 had a very low level of variation. Of all the 207 and 159 amplified fragments, 104 were polymorphic among the 16 plants of PI 577746 while only 11 were polymorphic in Hiverna/2-5EP1 (Table 3). The diversity value and Shannon's information index of two entries were also estimated as 0.171 and 0.253 for PI 577746 and 0.011 and 0.018 for Hirverna/5-EP1, respectively.

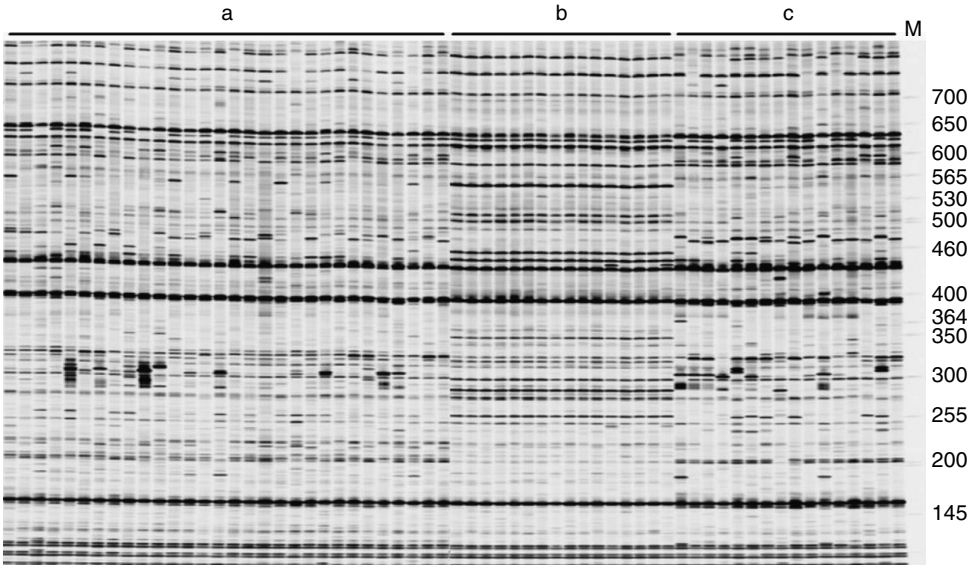

Fig. 3. TRAP profile of selected faba bean entries with primer combination MIR159A + Sa4-700. (a) Amplified from the bulked DNA of four random plants from each accession; (b) and (c) amplified from DNA of individual plants in Hiverna/2-5EP1 (b) and PI 577746 (c); M, the IRDye® 700 Sizing Standard 50–700 bp from LI-COR Biosciences (Lincoln, NE, USA).

Discussion

Comparing the two published reports using AFLP, with our results showed that TRAP is as effective as AFLP for revealing DNA polymorphisms for fingerprinting and assessing genetic relationships among faba bean genotypes. Zeid *et al*. (2003) used eight selected primer combinations and generated 527 fragments with an average of 65.8 fragments per primer combination. Zong *et al*. (2009) used ten primer combinations and generated 323 fragments with a mean of 32.3 fragments per primer combination. It is worth mentioning that six of the ten primer combinations were the same as the selected ones in the Zeid *et al*. (2003) paper. Thus, in AFLP and other kinds of DNA-based marker systems, the number of amplified fragments depends on the genotype used. In our current study, 221 fragments were amplified with six sets of PCR, or 36.8 fragments per PCR amplification. The percentages of polymorphic fragments in the two previous reports were 90.5 and 82.4%, respectively, while the number of the current study was much lower, only 55.2%. From Table 2, we can see that there is a great range in the percentage of polymorphic fragments among primer pairs, from 14.3% (MIR156A + Ga5-800) to 69.6% (MIR159A + Sa4-700). Therefore, it is possible to improve the efficiency of TRAP by adding a preliminary screening for primer combinations, which amplify more fragments and higher percentage of polymorphic fragments from the experimental materials. As in DNA sequence technology advances, more laboratories will adapt the high throughput, sequence-based fingerprinting platform for germplasm characterization in the future. However, there is currently a high cost associated with those platforms. Thus, TRAP offers an easy-to-perform and low-cost alternative for moderate through-put germplasm fingerprinting. Another advantage of TRAP is that the fixed primer can be designed against the sequences of the candidate genes involved

Table 3. Comparison of average genetic diversity and percentage of polymorphism among 16 individual plants from an inbred line and an open-pollinated accession

Entry	Number of plants	Total number of amplicons	Number of polymorphic amplicons	Polymorphism (%)	Diversity	Shannon's information index
Hervina/2-5EP1	16	159	11	5.0	0.011[a]	0.018
PI 577746	16	207	104	47.1	0.171	0.253
Mean				26.0	0.091	0.136

[a]Significance value: $P < 0.001$.

in controlling the phenotypes of interest to amplify the fragments associated with the phenotype. This has been documented by Miklas *et al.* (2006) for disease resistance trait in common beans, by Alwala *et al.* (2006) for sugar content in sugarcane and by Yue *et al.* (2010) for the ray flower color in sunflower.

Numerous reports on genetic diversity assessment involved genotyping a representative DNA sample prepared from bulked tissue from several individuals of an accession because this bulking strategy increases the efficiency of germplasm characterization. However, it is difficult to determine how many individuals to be used for each bulk. Fu (2003) summarized that from the 81 papers published during 1997–2002, approximately an equal number of researchers were in the following three categories: (1) using more than ten plants per bulk, (2) using six to ten plants per bulk and (3) using less than six plants per bulk. In the current study, we used only four plants per bulk to represent an accession. This number seems too small considering the high level of intra-accession variation in some of the accessions. However, faba bean has a large genome size of 12,000–13,000 Mbp, approximately 100 times larger than that of the model plant species *A. thaliana*. Using too many plants per bulk would make the scoring of some amplified fragments more difficult. It is unavoidable that the low-frequency markers will be amplified from the bulked DNA samples, but the genetic diversity and relationship among accessions should be based on common alleles rather than on rare alleles. The genotype data discriminated all the entries used in this experiment, which implies that our bulking strategy worked well.

Dice's (same as Nei and Li) coefficient, Jaccard's coefficient and the simple matching coefficient are the three commonly used coefficients in calculating similarity among accessions genotyped with molecular markers (Laurentin, 2009). Each of these coefficients has its unique property and none of them can overcome the disadvantage of dominant markers in dealing with heterozygosity in diploid or polyploid species. Several papers have discussed the suitability of particular coefficient to be used for particular marker data, but there is no consensus reached. Laurentin (2009) recommended the use of Jaccard's and Dice's coefficients and simple matching coefficient for dominant molecular markers in studies on non-related individuals and related individuals, respectively. In faba bean, Link *et al.* (1995) and Zeid *et al.* (2003) used Jaccard's coefficient for their AFLP data, while Zong *et al.* (2009) used the simple matching coefficient. We chose to use Dice's coefficient in the current study since this coefficient doubles the weight of the fragment presented in both individuals being compared for similarity. In an amplification-based marker system such as TRAP, the presence of the fragment is much more real than the absence of the fragment which could be produced by experimental errors. Therefore, the former should be emphasized as in the formula computing the Dice's coefficient.

It has been reported that various molecular techniques such as RAPD (Link *et al.*, 1995), AFLP (Zeid *et al.*, 2003; Zong *et al.*, 2009) and ISSR (Terzopoulos and Bebeli, 2008) were successfully used to assess the genetic variability among faba bean genotypes. The results we obtained from the current study are in good agreement with the previous reports. The distribution of faba bean cultivation is wide spread. There are numerous types of landraces or cultivars that have been selected to suit local environments in the production areas and to suit the end-use purposes. Therefore, it is not surprising that all three studies revealed a congruent association between molecular diversity and geographical origin of the entries under investigation. Zeid *et al.* (2003) reported that the eight Asian lines belonged to a distinct group separated from lines with other geographic regions such as Northern and Southern Europe and North Africa. Zong *et al.* (2009) reported that there was a high level of diversity among the 243 faba bean entries and that the entries collected from within China formed a separate distinct group from the entries from Europe, Africa and other parts of Asia. Our study confirmed that the faba bean germplasm from China are molecularly different from those from other parts of the world. The largest group in Fig. 1 has 101 Chinese entries. Those designated with prefixes of 'PI' were collected decades ago and those with prefixes 'W6' were collected more recently (Zhong *et al.* 2009). While the period at which faba bean moved into China is uncertain, one acceptable hypothesis is that faba bean was first introduced to the northern part of China from the Middle East 2100 years ago through the Silk Road in Central Asia (Zheng *et al.*, 1997). It is possible that the initial introduction was dispersed further to isolated areas in Southeast China and numerous landraces were developed by local growers.

Groups I to IV of Fig. 1 consist of accessions from two or more countries. The two entries in Group I were from Nepal and France. Most accessions in Group II were from Afghanistan, but one from Nepal and one from China were included. Four Chinese accessions and one Afghanistan accession formed Group III. Group IV has primarily European accessions and four Asian accessions. The possible explanation of the observed 'mixed' accessions in these groups is that these accessions moved from one place to another with human activities. The two commercial vegetable-type varieties were clustered in this group, which suggests that these two varieties were probably developed

from accessions closely related to European faba bean. Most of the bootstrap values on the cluster nodes (Fig. 1) are on the lower side. This could be the result of the high level of polymorphism revealed by TRAP markers and the level of heterozygosity caused by open pollination within some accessions. However, the two relatively high bootstrap values of 41 and 42 at the two major nodes separating Groups IV and V suggested that these accessions with Chinese and European origins were clearly divided and had less amount of gene flow among accessions between groups than within group.

Faba bean is a partially allogamous species. Outcrossing in faba bean landrace is facilitated by insect pollinators like honeybees (*Apis mellifera*), bumblebees (*Bombus* sp.) and diverse solitary bees (Bond and Kirby, 1999; Stoddard and Bond, 1987). As a result, the amount of outcrossing is quite high and variable. The documented mean was around 40–50% with individual records ranging from 4 to 84% based on morphological characteristics (Holden and Bond, 1960; Kendall and Smith, 1975; Poulsen, 1975; Bond and Poulsen, 1983; Link, 1990; Metz *et al*., 1992; Suso and Moreno, 1999; Suso *et al*., 2001, 2006, 2008). These large differences in outcrossing rate apparently were resulted from genetic and environmental factors as well as from the different methods used in their estimation. Recently, Gresta *et al*. (2009) reported that the estimated genetic variation of intraaccessions in each landrace and cultivar faba bean as *P*-distance ranged from 0.034 to 0.391 based on 364 AFLP fragments from five landraces. Terzopoulos and Bebeli (2008) revealed, with the aid of ISSRs, a high level of genetic diversity within the Mediterranean faba bean populations. Their finding that the average GS among bulked DNA samples within populations was 0.43 with a range from 0.21 to 0.676 implies a remarkable degree of intrapopulation diversity in their faba bean collection. In our current study, the 122 polymorphic TRAP markers displayed a significant difference between the two entries, PI 577746 and Hirverna/5-EP1, the percentage of polymorphism within the two groups being 47.1 and 5.0%, respectively (Table 3). This result demonstrated that TRAP is able to reveal the intra-accession diversity in faba bean germplasm collections. The accession PI 577746 not only possessed a high level of intra-accession variation at the molecular level but also segregated for flower color in the field. Considering the documented, very frequently occurring self-incompatibility in natural faba bean populations (Rowlands 1964; Terzopoulos *et al*., 2008), we assume that a substantial amount of genetic variation exists within faba bean landraces or within open-pollinated varieties such as PI 577746. Such variation has profound implication in practical breeding and should be further exploited in developing synthetic varieties to improve faba bean productivity. On the other hand, breeders have been attempting to breed self-compatible and self-pollinated inbred lines in order to produce hybrid varieties in the future, which will improve and stabilize the yield of faba bean crop. The obvious progress in the developing inbred faba bean lines has been evidenced by the observation of low polymorphism within the breeding line, Hirverna/5-EP1. However, there have been no stable cytoplasmic male sterile lines in faba bean for hybrid production (Link, pers. commun.).

The detected high level of genetic polymorphism within accession is of particular relevant to *ex situ* faba bean germplasm management, particularly with respect to the maintenance of such genetic variability. The following normal practices should be emphasized for faba bean germplasm conservation: (1) an optimal storage condition should be provided to collected seed samples to prolong the life span and to reduce the number of cycles for regeneration, (2) use a relatively larger population during necessary regeneration or seed increasing to mitigate the loss of genetic variation due to genetic drift and (3) protection from cross-pollination between accessions (Duc *et al*., 2010).

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